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The importance of kinase-phosphatase integration: lessons from mitosis

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Abstract

Kinases and phosphatases work antagonistically to control the behaviour of individual substrate molecules. This can be incorrectly extrapolated to imply that they also work antagonistically on the signals or processes that these molecules control. In fact, in many situations kinases and phosphatases work together to positively drive signal responses. We explain how this 'cooperativity' is critical for setting the amplitude, localisation, timing and shape of phosphorylation signals. We use mitosis to illustrate why these properties are important for controlling mitotic entry, sister chromatid cohesion, kinetochore-microtubule attachments, the spindle assembly checkpoint, mitotic spindle elongation and mitotic exit. These examples provide a rationale to explain how complex signalling behaviour could rely on similar types of integration within many other biological processes.

Introduction

The traditional view of protein phosphorylation is that kinases and phosphatases work antagonistically to switch individual signals on or off. It is an image that accurately describes the inner workings of all protein phosphorylation networks at their most fundamental level, i.e. an individual substrate molecule or residue. The problem is that this basic building block, which is merely a simple binary switch, is used by cells to output responses that are far more complex than that. This complexity arises because each individual molecule is often present in thousands of identical copies that must work together in time and space to collectively define the response. Imagine each copy as a separate light, and then visualise the different patterns or intensities that could be output within the cell and how this could change over time. Pulses, waves, flickering patterns, gradients of light, perhaps bursting into life in specific places at certain times. It depends what the signal requires: kinases and phosphatases may work in opposition to flick each light on and off, but they also work very much together to direct the bigger picture and define how the story unfolds.

This review uses specific examples from the field of mitosis to highlight key features of a signalling response that rely on cooperativity between kinases and phosphatases. We discuss each of these properties in turn from a systems perspective to rationalise why kinase-phosphatase integration is important, before providing examples where this has been shown to be the case. In each of these situations, kinases and phosphatases work together to define the signal and produce the correct type of response. This challenges the common belief that phosphatases mainly switch outputs off and suggests that they take a much more active role in transducing intracellular signals.

Signal amplitude

If an individual substrate molecule is a binary switch, which can be set to either 0 or 1, then the easiest way to generate signal intensities between 0 and 1 is to sense the collective behaviour of many of those individual molecules. The percentage of molecules that are on or off at any given time can therefore

determine the amplitude of the response. Kinase and phosphatases can work together in a wide variety of ways to reliably set this signal amplitude.

The simplest system to consider is one in which multiple copies of a basic building block (protein X) can be activated by phosphorylation (Figure 1A). If the signal input increases the kinase activity (Input = $Input_1$), the probability of finding an individual switch in the phosphorylated state increases as well. A typical time evolution of phosphorylation and dephosphorylation dynamics for constant kinase and phosphatase activity is shown in Figure 1B, both for one individual substrate (blue) and the average response of one hundred substrates (black). Assuming simple mass action kinetics, one finds a hyperbolic relation between the kinase activity (input) and the average phosphorylation state (Figure 1C) [1]. Exactly the same input-output relation is found if the signal input decreases the phosphatase activity by equal amounts (Input = $Input_2$). In other words, changing kinase or phosphatase activities have *equivalent* effects on the signal amplitude.

While the hyperbolic relation of Figure 1C allows the signal amplitude to be modulated over a wide range, it fails to get maximal phosphorylation for a wide range of input values. One mechanism to improve this response is to have the input increase the kinase activity, as well as decrease the phosphatase activity (Input = $Input_1$ + $Input_2$), a scheme called *reciprocal regulation* [2]. Such regulation turns the input-output response more switch-like and for similar input values a higher phosphorylation can be obtained (Figure 1D). This is a scheme that is used in mitotic entry: increasing activity of Cyclin-dependent kinase 1 (CDK1) also indirectly phosphorylates ENSA/ARPP19 (via Greatwall kinase), to turn it into an inhibitor of the opposing phosphatase PP2A-B55 [3-5]. As illustrated in Figure 1D, the steady-state input-output response is often sigmoidal, meaning it is flat at both high and low input, while it changes more abruptly in between. Such a response can be approximated by the well-known Hill equation (see Figure 1D), where the Hill coefficient n is a measure for the steepness or sensitivity of the response. When $n > 1$, the response is also said to be *ultrasensitive*. Apart from reciprocal regulation (n = 2), kinases and phosphatases can interact in a wide variety of ways to generate different levels of ultrasensitivity (For an excellent review series on this topic, see [1, 6, 7]). An even more efficient way to create a clear switch between minimal and maximal phosphorylation when changing the input signal is to include *feedback* between substrate, kinase, and phosphatase. For example, if the phosphorylated protein X increases the input kinase activity, this further increases its own phosphorylation. Similarly, if it decreases inhibitory phosphatase activity, this limits its own dephosphorylation. Such feedback mechanisms have been shown to lead to *bistability* in the input-output response [7-12], producing a region of input values where one can have both minimal and maximal phosphorylation of the substrate (see highlighted region in Figure 1E). For a given input within this bistable region, any initial stimulus below a threshold value (black) will evolve to a stable state of low phosphorylation (blue). Similarly, if the system initially finds itself above this threshold, it will relax to a state of high phosphorylation (red). Moreover, as there is a wide range of input values that lead to minimal or maximal phosphorylation, the output is robust to small changes in input signal. Finally, changing the input across the highlighted bistable region leads to a switch from minimal to maximal phosphorylation, without settling at intermediate phosphorylation states. This type of regulation is known to be critical at different stages of mitosis. For example, robust mitotic entry is based on a bistable switch [12-14], where CDK1 inhibits its inhibitory kinase Wee1 [15-18] and also activates its activating phosphatase CDC25 [19-21]. Similarly, Aurora B activates its activating kinase Haspin but also restrains its inhibitory phosphatase PP1-RepoMan [22, 23].

These examples highlight how signals can avoid resting in a state of submaximal phosphorylation. However, in some instances, this may be exactly what is needed. For example, imagine a signal that must respond to different inputs by increasing or decreasing the output accordingly. In this case, additional stable steady states of submaximal phosphorylation are required. Multisite phosphorylation where the involved kinase and phosphatase are both saturated by an excess of substrate is one way to achieve this [24], and changing either enzyme activity can give access to stable phospho-forms of the substrate that correspond to

different submaximal activities. It is not yet clear whether such a mechanism is used in mitosis, but it may play a role in the attachment of microtubules to kinetochores.

Kinetochores are specialised multi-protein complexes that assemble on sister chromosomes and allow their attachment to microtubules during mitosis [25]. The major microtubule binding component of the kinetochore is NDC80, which is able to capture microtubules that generate tension whilst releasing ineffective attachments that don't [26]. This is possible because the tension-sensitive kinase, Aurora B, phosphorylates up to nine residues in NDC80 to reduce microtubule affinity [27-29]. Importantly, it is the cumulative number of phosphorylated sites that determines the affinity of NDC80 for microtubules, and therefore NDC80 operates like a rheostat that can fine-tune microtubule affinity in a graded manner [30]. Not surprisingly, inhibiting Aurora B or its antagonising phosphatase, PP2A-B56, swings the system to a fully off or on state where microtubules are either stably bound or completely unattached [31-36]. This implies that the amplitude of NDC80 phosphorylation is important and that it likely exists primarily in a state of submaximal phosphorylation to allow microtubule affinity to be increased or decreased when required. It is still unclear whether localised Aurora B and PP2A-B56 are saturated by NDC80 at kinetochores, but if they are, then multisite phosphorylation could provide the stable intermediate levels of phosphorylation required.

Multisite phosphorylation is not the only way to achieve stable submaximal phosphorylation, and in some cases single phosphorylation sites also require strict amplitude control. A good example again involves the regulation of microtubules, but this time at the central spindle during mitosis. Overgrowth of microtubules in this region is prevented by the motor protein KIF4A, but only when it is phosphorylated by Aurora B [37]. PP2A-B56 is needed to antagonise this phosphorylation, otherwise microtubule dynamics are supressed too much and the resulting central spindles are abnormally short [38]. Therefore, this kinase-phosphatase pair works together to set the amplitude of KIF4A phosphorylation, which likely ensures that the system can respond swiftly to subtle changes in length of the overlapping microtubule bundle by increasing or decreasing microtubule polymerisation accordingly. How this type of regulation occurs is still unclear, but feedback from the output (e.g. length of central spindle and/or overlapping bundle) to the input (Aurora B and/or PP2A-B56) could offer one plausible mechanism.

The examples described above demonstrate some different ways in which the amplitude of phosphorylation signals can be regulated, however, this list is by no means exhaustive. Whereas we mainly focus on the simplest case of a single phosphorylation site controlling a protein's function, it is clear that in practice, there will often be multiple phosphorylations on a single substrate. Although such a system can still behave in a very similar manner, it can also introduce additional complexity [6, 24]. The key point that we want to convey here, however, is that in all these cases the level of phosphorylation is set by the balance of activities between kinase *and* phosphatase. This balance could allow a signal to change gradually, as kinase or phosphatase activities rise and fall, or switch abruptly, as a consequence of kinase-phosphatase feedback and/or multisite phosphorylation. This ability to reliably change signal amplitudes is not only important for defining key mitotic transitions, such as the ones illustrated above, it is also critical for the spatial control of signalling.

Spatial organisation

There are many classical examples of signalling events that must be confined to particular areas of the cell. The centrosome, kinetochore and central spindle, for example, are all subcellular structures that perform specialised roles during cell division which, not surprisingly, critically depend on localised signalling events. One of the simplest ways to generate such localised responses is to only place the substrate exactly where it is needed. The kinetochore signalling scaffold protein KNL1 is an excellent example [39]. However, if the same substrate is also required in different areas of the cell at the same time, there need to be more subtle ways of achieving subcellular specificity. In some cases, relocalisation of the upstream kinase can suffice, but this alone would be error-prone if kinase activity could not be completely restricted to the required site

of action. In this case, phosphatases can be used to remove any surplus phosphorylation from these inappropriate locations. Although this could in principle rely on a basal level of phosphatase activity throughout the cell, there are well-documented examples where kinase and phosphatase activities are intrinsically entwined to ensure that the kinase-phosphatase balance can switch abruptly over nanometre scale distances.

Figure 2A sketches a situation where kinase activity dominates over phosphatase activity in the centre of a domain, while the reverse is true in the rest of the domain. If we assume that a substrate does not diffuse (e.g. because it is strongly bound to an organelle), its phosphorylation state will be determined by the local kinase and phosphatase activities. If the differences in kinase-phosphatase activities are large enough, the centre of the domain will be close to maximal phosphorylation, while the rest of the domain will be minimally phosphorylated (Figure 2B; see also Section 1: Signal Amplitude). In mitosis, such specific substrate (de)phosphorylation through kinase and phosphatase coordination is commonly found at centromeres.

A first example is illustrated in Figure 2C. The duplicated sister chromatids remain physically connected through a ring-like structure called cohesin during S/G2 phase. In animal cells, immediately following mitotic entry, the bulk of this cohesin is removed from chromosome arms [40]. This loss of cohesion on the arms is tolerated because the cohesin complex is still maintained at the centromere, which is sufficient to resist the pulling forces exerted by microtubules. Regulation of the kinase/phosphatase balance along chromosomes underlies this spatial control of cohesion. In short, along the chromosome arms, mitotic kinases (Aurora B, PLK1 and CDK1) cooperate to phosphorylate key cohesin subunits and regulators (SA2 and Sororin) [41, 42], whilst also inhibiting an antagonizing phosphatase (Ssu72). Together, this enhances phosphorylation to permit the opening of cohesin rings [40, 43]. In contrast, at the centromeres these kinase inputs, directly or indirectly, *promote* the accumulation of the antagonising phosphatase complex PP2A-B56-Sgo1, which restricts phosphorylation locally to preserve cohesion [40, 44-47]. This demonstrates how spatially confined regions of positive or negative regulation, whereby kinases either inactivate or activate phosphatases, can lead to different outputs.

This centromeric enrichment of cohesion also helps to enrich Aurora B at centromeres, where it is needed to phosphorylate key substrates that regulate kinetochore-microtubule attachments. Aurora B is part of the Chromosomal Passenger Complex (CPC) and key subunits of this complex bind to Sgo1 and histone H3 tails that are phosphorylated at Thr3 (H3T3) [48-50]. Both of these recruitment axes are enriched by cohesion: Sgo1 interacts with cohesin, as does the H3T3 kinase, Haspin [46, 48, 51, 52]. This initiates Aurora B recruitment, which further reinforces its own centromeric accumulation by locally activating Haspin [23, 53] and opposing the recruitment of the H3T3 phosphatase PP1-RepoMan [22] (Figure 2D). This second example illustrates how feedback, that can give rise to bistability (Figure 1C), can be used to cause abrupt changes in localised signalling events. Furthermore, taken together, these two examples of Aurora B and cohesin regulation illustrate beautifully how multiple kinase-phosphatase feedback loops and feedforward interactions can be intertwined to provide strict spatial signalling control.

Now let us consider a situation when the kinase-phosphatase ratio is still maintained in the two domains sketched out in Figure 2A, but the substrate is able to diffuse freely (see Figure 2E). The substrates X will be fully phosphorylated in the centre of the domain, but then diffuse away into the domain where the phosphatase is dominant, become dephosphorylated, and only get phosphorylated again if they reach the centre once more via diffusion. This dynamic situation equilibrates after a while and leads to the creation of a substrate phosphorylation gradient. The maximal phosphorylation is determined by how dominant the kinase is over the phosphatase in the central domain, while the phosphorylation level decays exponentially outside of the center according to $e^{-x/L}$, $with\ L = \sqrt{D/k_{dephosph}}$ [8, 9, 54]. This length L is a measure for how deep the phosphorylated substrate can penetrate the outer domain before becoming dephosphorylated. It increases with its diffusion coefficient D (assumed equal for phosphorylated and

unphosphorylated substrate) and decreases with the rate of dephosphorylation $k_{dephosph}$. The shape of such a phosphorylation gradient can be further changed by the underlying biochemical networks (phosphorylation cascades are, for instance, one way to increase the penetration depth [54]), dimensionality or differences in diffusion coefficients [8, 9, 54]. Finally, kinases and phosphatases can themselves also be substrates to create additional layers of complexity.

One well studied example of such an intracellular gradient in the context of mitosis is the cortical gradient of Pom1 kinase in fission yeast, which has been proposed to measure cell length by inhibiting the mitotic activator Cdr2 [55, 56]. Pom1 concentrates at the cell tip and decreases toward to the cell centre, where Cdr2 is located (Figure 2F). The longer the cell grows, the more likely it is to divide because less Pom1 is localized at the cell centre, which allows the activation of Cdr2 to trigger the cell division machinery [56]. At least two properties are required for the formation of this gradient. First, a diffusion based-mechanism ensures that active Pom1 is released from membranes following autophosphorylation. Second, Pom1 interacts with a PP1-Tea phosphatase complex, which can dephosphorylate Pom1 and restore its ability to bind the plasma membrane. Crucially, this PP1-Tea complex is delivered to the cell tip by microtubules that are aligned along the growth axis, which dephosphorylates Pom1 locally and replenishes the 'source' of the Pom1 gradient [56-58]. This shows how an intracellular gradient can be built by integrating kinase-phosphatase signaling along with a polarity cue.

Collectively, these examples demonstrate how the correct type of localised response relies on integrated signalling between *both* kinase and phosphatase. In some cases, this enables the control of both signal amplitude *and* localisation, which allows more complicated outputs such as spatial gradients to form. This complexity is increased even further when we consider that none of these processes occur instantaneously. The speed and exact timing with which changes in signal amplitude occur at different locations critically depends on kinase-phosphatase integration as well.

Temporal organisation

There is perhaps no better process to illustrate the importance of signal timing than mitosis. The tightly coordinated sequence of events that typify cell division are only possible because signalling activities are able to abruptly rise and fall in a timely ordered manner. One might incorrectly assume that this is not reliant on kinase-phosphatase 'integration', because these enzymes simply turn signals on or off independently at the appropriate times. However, the timing aspect of signalling (i.e. how fast and in what order signals are switched on or off), is often critically dependent on kinase-phosphatase cross-talk.

The speed of signal switching

Let us start with the issue of speed and how quickly signals can be switched on or off. The simplest scenario is to imagine a substrate X that is activated ('on') by a kinase and inactivated ('off') by a phosphatase. When 'off', the rate of activation will be quicker in the absence of phosphatase (Figure 3A), but when 'on' the rate of deactivation will be quicker if the phosphatase is primed and ready to dephosphorylate (Figure 3B). A simple way to achieve this is to allow the phosphatase to be recruited by the phosphorylated substrate, such that it is disengaged when the signal is 'off' but then recruited and primed when the signal is 'on'.

This type of arrangement controls the spindle assemble checkpoint (SAC) at kinetochores, which delays chromosome segregation until all sister chromosomes are properly attached to the mitotic spindle [59]. The kinase MPS1 phosphorylates KNL1 on 'MELT' repeats to recruit various checkpoint components to the kinetochore and activate the SAC [60-62]. One of these components, BUBR1, also recruits a phosphatase, PP2A-B56, which dephosphorylates the MELTs (indirectly via PP1) [34-36, 63, 64]: PP2A-B56 actually antagonises Aurora B to dephosphorylate an 'RVSF' motif that is needed to recruit PP1 to kinetochores [63, 65] (Figure 3C). Why would the SAC signal also recruit a phosphatase that tries to silence that signal? One possibility, is that this arrangement allows the SAC to start-up and shut-down as quickly as possible. Firstly, the phosphatases are disengaged for start-up, because BUBR1 is excluded from the nucleus in prophase,

and therefore the kinase can activate the signal unopposed. Secondly, after nuclear envelope breakdown, the phosphatases are then primed to shut down the signal rapidly when kinetochore microtubules attach correctly and kinase activities are inhibited (Figure 3D). Therefore, intricate coupling between kinase, phosphatase and substrate is important to ensure the checkpoint signal can switch rapidly on or off.

The timing of signal switching

Another aspect to signal timing, which relates to accuracy more than speed, is when signalling events must be precisely ordered. During mitosis, a specific kinase-phosphatase pair may coregulate multiple different substrates, and yet sometimes the (de)phosphorylation of these substrates must occur in a temporally ordered manner. The dephosphorylation of CDK1 substrates upon mitotic exit is an excellent case in point. Figure 3E and F sketches two general ways by which substrate dephosphorylation can be ordered in time in response to a signal that leads to a strong decrease in the kinase - phosphatase activity ratio. As in Figure 1, we refer to this ratio as the input of the system and we assume that the input-output response is very ultrasensitive (see Figure 1D: high value of *n* with a clearly defined threshold *K*).

First, in Figure 3E, the phosphatase becomes gradually more dominant than the kinase (see red curve), and we assume that two substrates (green and blue) have a *different threshold* (K_1 vs. K_2) for (de)phosphorylation in response to the activity of the kinase and phosphatase. Such threshold differences can couple the temporal changes in kinase and phosphatase activity to a clear timing difference in the onset of substrate (de)phosphorylation ($t_1 \neq t_2$). These thresholds can be simply set by differences in the specific rate at which individual substrates are phosphorylated or dephosphorylated by the same kinase or phosphatase; for example, differences in flanking amino acids, native protein conformation, binding affinities and subcellular localisation can all impact on the catalytic efficiency (K_{cat}/K_m) towards individual substrates.

Second, in Figure 3F, we assume that there is no difference in threshold ($K_1 = K_2$) and/or the input changes very abruptly, such that both substrates receive the signal to dephosphorylate at the same time ($t_1 = t_2$). Instead, the temporal ordering of substrate (de)phosphorylation is now established by different substrates having a *different time delay* to become (de)phosphorylated ($T_1 \neq T_2$). In Figure 3F, the green substrate ($Output_1$) is dephosphorylated after a short time delay T_1, while the blue one ($Output_2$) only responds later after a time delay T_2. Having cascades of sequential events, such as in substrates with multiple phosphorylation sites, is one way to create such a time delay [66, 67], while substrate competition is another [6].

These models are by no means mutually exclusive, and in fact, there is good evidence that both are used to coordinate substrate dephosphorylation during mitotic exit. In budding yeast, the order of substrate dephosphorylation is determined in part by the threshold model because kinase/phosphatase (CDK1/CDC14 [68] and CDK1/PP2A-CDC55 [69]) activity ratios differ towards individual substrates, with the phosphatase displaying a greater catalytic efficiency towards early substrates. This activity ratio can also determine when substrates are switched on, since in general, good kinase substrates are phosphorylated early and good phosphatase substrates are phosphorylated late [69-71]. Together, these studies demonstrate nicely how the intrinsic properties of each substrate can define exactly when that substrate will switch states in response to a defined kinase-phosphatase equilibrium. In addition to this, substrate competition also contributes to ordering by generating time delays because low efficiency (late) phosphatase substrates are dephosphorylated much later in the presence of high efficiency (early) substrates [68].

In mammalian cells, the major phosphatase complex that reverses CDK1 phosphorylations during mitotic exit is PP2A-B55, but this also relies on both the threshold and time delay models to order substrates. Firstly, PP2A-B55 is inhibited prior to mitotic exit by phosphorylated ENSA/ARPP19, which is an unusually poor substrate that has a very high affinity (Km = 1 nM) and low catalytic rate (Kcat = 0.05 sec^{-1}) [72]. It is

also in 5-fold stoichiometric excess over PP2A-B55, which creates 'unfair competition' with other substrates, such that the phosphatase is unable to act on these until ENSA/ARPP19 is fully dephosphorylated [72]. This provides a biochemical time delay that is necessary to allow sister chromatids to separate away from the division plane before PP2A-B55 can initiate cytokinesis [73]. Secondly, once PP2A-B55 is active it coordinates dephosphorylation of different substrates based on the threshold model. PP2A-B55 displays preference for substrates with phospho-threonine (as opposed to serine) that are also surrounded by polybasic patches of amino acids. This allows the motif of individual substrates to help to determine when they become dephosphorylated [74, 75]. Finally, it is important to also note that the function of the basic residues is to increase substrate residence time, owing to interaction with an acidic surface on PP2A-B55 [75]. This will, in turn, contribute to substrate competition and therefore substrate ordering via the time delay model as well.

Together, these examples serve to illustrate the importance of understanding how kinases and phosphatases co-regulate substrates. Even when they appear to act independently to start up or shut down signals, the balance and integration of their respective activities can determine the speed and timing by which those signals are initiated or silenced. We would like to now move on to discuss how kinases and phosphatases can work together to control various combinations of all of the above. We have already discussed how amplitude and localisation can be combined to give rise to spatial gradients, but the addition of time adds another layer of complexity that, together, can be used to define signal shape.

Shaping signals in time and space

How a signal behaves over time can be critical for the overall response: Figure 4A shows a network structure that uses a single input to drive a more complex biphasic output X. The input activates both a kinase and a phosphatase, but the activation of the phosphatase is delayed. While the kinase is immediately able to phosphorylate the substrate X, the phosphatase only starts dephosphorylation after a time delay. Such an incoherent feedforward loop can generate a biphasic response in X [76], and this type of regulation is known to be important during meiosis I [77]. The bi-orientation of bivalents precedes the stable attachment of microtubules to kinetochores. This order is important because premature stabilization of microtubule attachments can generate daughter cells with an abnormal number of chromosomes. The key to this timing module is the slow but progressive activation of CDK1 [77]. Low CDK1 activity is sufficient to activate Aurora B/C and disrupt erroneous kinetochore-microtubule interactions (via NDC80 phosphorylation), whereas higher levels of CDK1 activity cause the additional recruitment of PP2A-B56 to counteract Aurora B/C and stabilize these interactions (Figure 4B). Thus, CDK1 both inhibits and stabilizes the binding of microtubules to the kinetochores, and the biological output is determined by threshold at which Aurora B/C and PP2A-B56 respond to CDK1 activity. This essentially allows a slow rise in CDK1 activity to control a transient, biphasic phosphorylation of NDC80 [77]. This example demonstrates how kinase and phosphatase can work together to change signal amplitude over time. However, there are more complex situations when amplitude, localization and timing can all change due to kinase-phosphatase cooperativity.

A good example is provided by spatial waves [78-80]. Let us illustrate this for the pool of Aurora B that is concentrated at the centromeres by the counteracting activities of Haspin and PP1-RepoMan (Figure 2D). The centromeric Aurora B complex is activated by CDK1 phosphorylation and autophosphorylation (Figure 4C) [81-83]. Activated Aurora B diffuses away from the centromeres and generates a phosphorylation gradient [84]. This creates a 'mixed region' of intermediate Aurora B activity close to the centromeres. If this activity of Aurora B is higher than the bistability threshold, indicated by the black dashed line, then the network interactions will cause that mixed region to rapidly switch to a state of high Aurora B activity. This causes a snowball effect of 'mixing' of the high and low activity regions through diffusion, and activation of this mixed region due to bistability of the network. As a result, instead of forming a spatial gradient, diffusion leads to a sharp spatial phosphorylation profile that spreads with a fixed velocity v from the centromeres to the chromosome arms (Figure 4C). In other words, after every time Δt, the high activity state propagates over a distance $L = v\,\Delta t$ and this wave does not diminish in speed or amplitude [78-80].

Such wave propagation of Aurora B has effectively been observed *in vivo*, as well as *in vitro* using a reconstructed kinase-phosphatase switch regulating Aurora B [84, 85]. The explicit influence of PP1-RepoMan and Haspin have, however, not yet been explored. Similarly, mitotic entry has also been shown to spread via spatial waves of CDK1 activity that result from an underlying bistable switch involving an inhibitory kinase Wee1 and an opposing activating phosphatase CDC25 [12, 78, 86, 87].

In both of these examples, kinase-phosphatase integration allows the amplitude of a signal to increase and spread out over time. The resulting wave is important to rapidly distribute activity away from a point source, however in other contexts it can be important to do the exact opposite, and restrict activity back towards the source. Not surprisingly, here too, kinase and phosphatase cooperativity is critical. Chromosome segregation is controlled by a gradient of Aurora B activity that reaches out from the centromere to inhibit microtubule attachments at the kinetochore. This activity must selectively remove incorrect microtubule attachments that fail to generate tension, whilst leaving the correct, tension-generating types, intact. This process, known as error-correction, relies on an intricate feedback circuit involving at least two kinases (Aurora B and BUB1) and two phosphatases (PP1 and PP2A-B56) [88]. It is likely that this circuit allows the gradient of Aurora B activity to narrow rapidly in time when tension is exerted across the kinetochore, which in turn helps to limit Aurora B activity and prevent the inappropriate removal of correct kinetochore-microtubule attachments (Figure 4D).

Concluding remarks

We have illustrated here how kinases and phosphatases work together to control the amplitude, localisation, timing, and shape of phosphorylation signals. These are all critical features of a signaling response, and therefore, in many situations these enzymes must work together to positively drive the correct output. We have highlighted this point using examples from mitosis because here the relevant kinases, phosphatases and substrates are well characterized and the output signals can be readily quantified. However, it seems likely that similar types of co-regulation will also exist within other cellular processes that rely on protein phosphorylation. To address this point a number of challenging questions must first be answered (see outstanding questions). An important first step is to better characterize the specific phosphatase inputs for each of these processes. This has been somewhat hampered by the difficulties associated with inhibiting these inputs specifically: phosphatases lack a well-defined pocket for small molecule inhibitors (such as the ATP pocket in kinases) and their catalytic subunits are often shared between many different holoenzyme complexes [89]. However, these problems have been alleviated of late by the discovery that phosphatase holoenzymes can be specifically inhibited by targeting their regulatory subunits with small molecules [90] or point mutations [63, 91, 92].

Armed with tools to interfere with phosphatase activity specifically, it is then important to ask how these inputs work together with the kinases to shape the output in the desired way. At this point a key question becomes one of cross-talk. How do these antagonistic enzyme pairs regulate each other and what is the significance of that interplay? The phosphatase recruitment motifs are often regulated by kinases: Binding of the RVxF motif to PP1 is inhibited by phosphorylation [93], for example, whereas binding of the LxxIxE motif to PP2A-B56 is enhanced by phosphorylation [92]. Clearly the kinases themselves are also regulated by phosphorylation and therefore subject to feedback regulation from phosphatases. Finally, if all this occurs on a localised scale, then a key step will be to identify and characterise the scaffold proteins that mediate this cross-talk. Two of the proteins highlighted in this review, KNL1 and RepoMan, are scaffold proteins that allow the activities of multiple kinases (CDK1, Aurora B, MPS1) to converge on multiple phosphatases (PP1 and PP2A-B56) to produce the desired output during mitosis [22, 63, 94]. There have been many excellent reviews on scaffold proteins within other cellular processes that mediate similar types of integration [95-97]. Some of the principles discussed here may also be relevant in these contexts.

Finally, once the molecular connections that integrate kinase and phosphatase signals have been defined, it is then crucial to determine what those connections mean for the output of the response. Do they regulate individual properties such as amplitude, localisation or timing, or do they control multiple properties together to produce more complex outputs, such as spatial gradients (amplitude and localisation) or spatial waves (amplitude, localisation and timing)? To answer these questions, it will be important to adopt a holistic approach to interrogate the network as a whole, using systems biology for example, instead of simply studying its individual components in isolation. Ideally, this can be combined with biochemistry and/or synthetic biology to attempt to build the network from its constituent parts.

In summary, as pointed out recently by others [98], it is becoming increasingly clear that enzymes that have traditionally been viewed as 'negative' regulators can in fact have much more 'positive' roles in cell signaling. We expand on this point here to show that, for protein phosphorylation at least, antagonistic enzyme pairs can be more important than either enzyme alone. In all the examples discussed above, the correct signal output critically depends on both enzymes working together. It is tempting to speculate that other types of reversible posttranslational modifications could similarly depend on co-regulation from enzyme pairs that are traditionally viewed as 'antagonistic'. Histone acetylation, for example, needs to be tightly regulated in time and space to control gene expression. This could require cross-talk between histone acetyltransferases (HATs) and deacetylases (HDACs) [99], or between alternative enzyme pairs that can co-regulate HAT and HDAC activity [100]. It will be important in the future to determine just how important antagonistic co-regulation is for these alternative modes of signaling.

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Figure legends

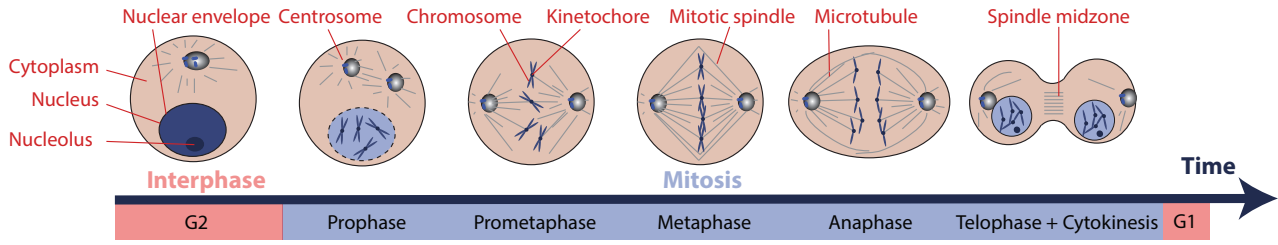
Box 1. An overview of the kinases, phosphatases and other proteins that regulate mitosis. The processes, kinases, phosphatases and localisations depicted are all discussed within this review. They are not, however, meant to be an exhaustive list.

Figure 1: Signal Amplitude. The signal amplitude (Output = phosphorylated protein X) can be regulated by changing the kinase and/or phosphatase activity (Input) in various ways. **(A)** Sketch of simple antagonistic regulation**. (B)** Time evolution of the phosphorylation state of a single substrate X (blue), as well as the average response of 100 substrates (black). A Gillespie algorithm is used to simulate the antagonistic regulation in (A) using the following parameters: the rate of kinase activity $k_f = 1$, the rate of phosphatase activity $k_b = 1$. a.u., arbitrary units. **(C)-(E)** Depending on the type of kinase-phosphatase integration, the input-output characteristics can be hyperbolic (C); ultrasensitive (D); or multistable (e.g. bistable). In these examples $K = k_f / k_b = 1$ and Input = Kinase activity / Phosphatase activity. Phosph., phosphorylation.

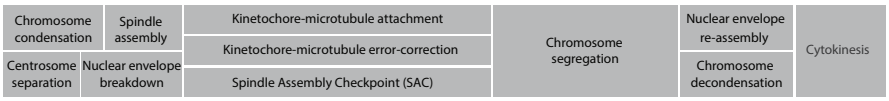
Figure 2: Spatial organisation. A. Sketch showing a central region with a high kinase/phosphatase ratio (kinase dominant), surrounded by two regions with a low kinase/phosphatase ratio (phosphatase dominant). Kinase is indicated in red, phosphatase in blue. **B.** Outline of the spatial distribution of average phosphorylated substrate X in response to the kinase and phosphatase distribution in (A) if the substrate cannot diffuse. **C,D.** Two examples from mitosis where the kinase/phosphatase ratio changes in space. This leads to; (C) cohesin removal from chromosome arms but not from centromeres, or (D) H3T3 phosphorylation and Aurora B activation at centromeres but not on chromosome arms. **E.** Same as (B), but with substrate diffusion, such that a spatial gradient is formed. **F.** An example from mitosis of how an intracellular cortical gradient in the kinase Pom1 is formed in fission yeast. Pom1 autophosphorylation drives its own release from membranes. Microtubules deliver a Tea-PP1 complex along the growth axis towards the cell tip, which allow localized Pom1 dephosphorylation and membrane rebinding specifically at the cell tip.

Figure 3: Temporal organisation. A. Signal activation is fastest in the absence of a counteracting phosphatase. However, the phosphatase might be needed to ensure the correct final signal amplitude. By delaying phosphatase activation, the system can be responsive, as well as relax to the desired signal amplitude. Simulations use simple antagonistic regulation (Fig. 1(A)) with $k_f = k_b = 1$, kinase = 10, and phosphatase goes from 0 to 5 at the mentioned times (0, t_1, t_2, never). **B.** Signal deactivation is faster in the presence of additional phosphatase. Simulations again use antagonistic regulation (Fig. 1(A)) with $k_f = k_b = 1$, kinase goes from 10 to 0 at time = 1, and phosphatase = 1 (red), 5 (black). **C.** At unattached kinetochores in prometaphase, SAC signalling is initiated by MPS1-dependent MELT phosphorylation. This primes PP2A-B56 phosphatase to silence that signal via PP1. Aurora B prevents premature silencing by phosphorylating the RVSF motif to limit PP1 recruitment to kinetochores. **D.** In prophase, SAC initiation by MPS1 is unopposed by counteracting phosphatases because BUBR1 is excluded from the nucleus. This allows rapid phosphorylation of the MELT motifs. In prometaphase, following microtubule attachment, kinase activities are inhibited and phosphatases are primed to rapidly dephosphorylate the RVSF and MELT motifs. **E, F.** Substrate (de)phosphorylation can be ordered by combining different phosphorylation thresholds with non-instantaneous changes in the kinase/phosphatase Input ratio (E), or by having different response times (delays) when crossing the (de)activation threshold (F).

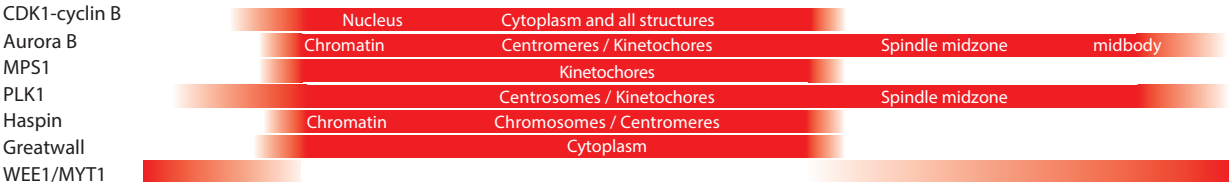
Figure 4: Shaping signals in time and space. A. An incoherent feedforward loop can lead to differences in activation time of a kinase and its opposing phosphatase, creating a biphasic timer. **B.** Such an incoherent feedforward loop is used in the regulation of NDC80 during meiosis. **C.** Sketch illustrating how bistability and diffusion of the substrate can give rise to spatial trigger waves through consecutive cycles of mixing (gray) of a high state (red) and low state (blue), followed by this mixed region quickly relaxing to the high state. Aurora B phosphorylates histone H3 at Ser10 (H3S10). **D.** Sketch illustrating how an Aurora B gradient at centromeres/kinetochores could be reduced upon kinetochore-microtubule attachment.



Cell processes



Kinase activity



Phosphatase activity

Kinases

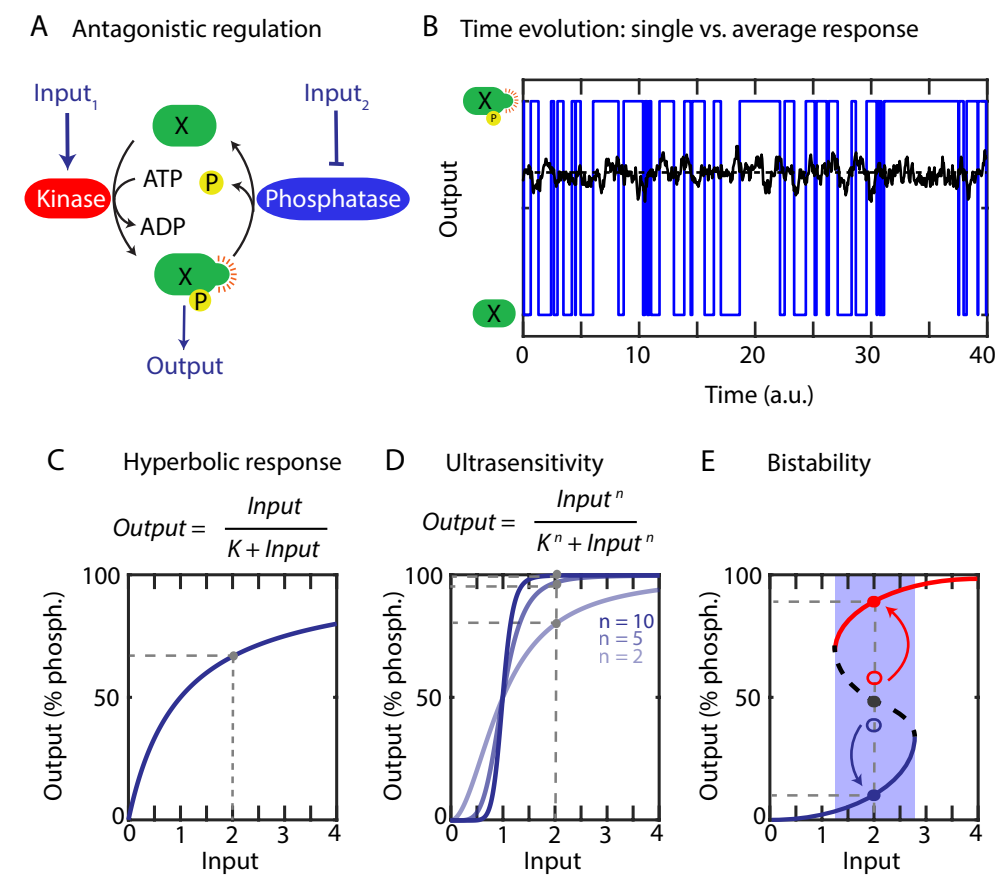
CDK1-cyclin B	The Cyclin-dependent kinase 1 - cyclin B complex triggers mitotic entry through phosphorylation of numerous proteins.
Aurora B	The active component of the chromosomal passenger complex (CPC). It contributes to the initiation of the spindle assembly checkpoint (SAC) and disrupts improper interactions between microtubules and kinetochores. During (pro)metaphase and anaphase the CPC is enriched at centromeres and the spindle midzone, respectively.
PLK1	Polo-like kinase 1 plays an important role in centrosome maturation and the establishment of a bipolar spindle. Among its substrates are CDC25C and APC/C, which are both activated by PLK1.
Haspin	Contributes to centromeric targeting of the CPC during prometaphase through phosphorylation of histone H3 at Threonine 3.
Greatwall	Turns endosulfine α (ENSA/ARPP19) into an inhibitor of PP2A-B55, and thereby contributes to a timely mitotic entry.
WEE1/MYT1	Inhibits mitotic entry by inhibiting the activity of CDK1-Cyclin B through phosphorylation of CDK1 at Tyrosine 15.
MPS1	Initiates the spindle assembly checkpoint (SAC). Its key substrates are the kinetochore proteins BUB1 and KNL1.
BUB1	Recruits BUBR1 and other SAC regulators to kinetochores by binding to KNL1. It also phosphorylates histone H2A to recruit Aurora B to the centromeres.

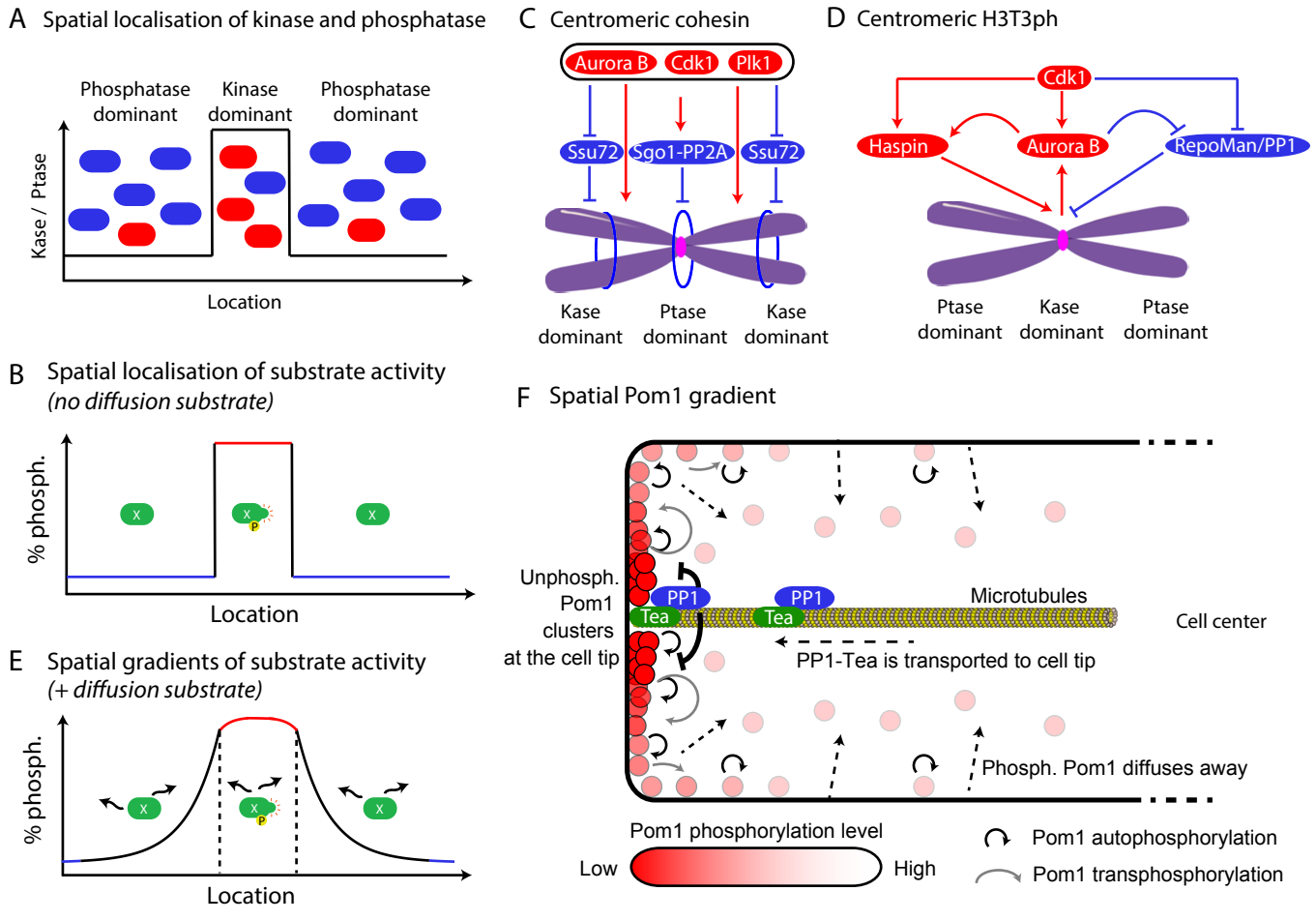
Phosphatases

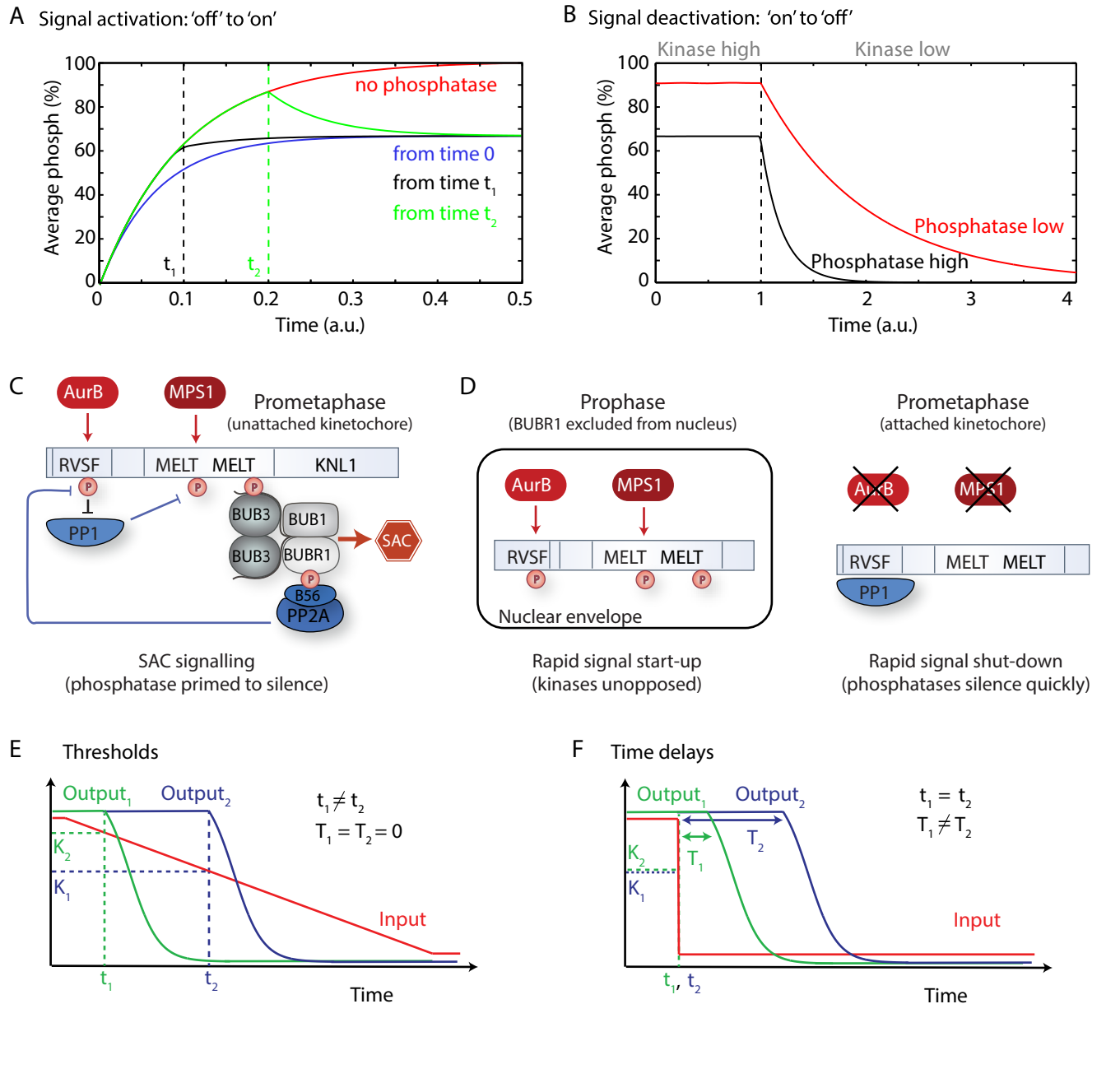
CDC25	A dual specificity protein phosphatase that removes inhibitory phosphorylations from CDK1.
PP1	Ser/Thr phosphatase that forms heterodimeric complexes with many PP1-interacting proteins, which determine its specificity and activity. PP1 holoenzymes have various functions throughout mitosis.
PP2A	Ser/Thr phosphatase that forms heterotrimeric complexes with a scaffolding A subunit and a regulatory B-subunit. The most important mitotic B-subunits are isoforms of B55 and B56. PP2A holoenzymes have many substrates throughout mitosis.
CDC14	Dual specificity phosphatase that is best known for its contribution to the mitotic exit in yeast.

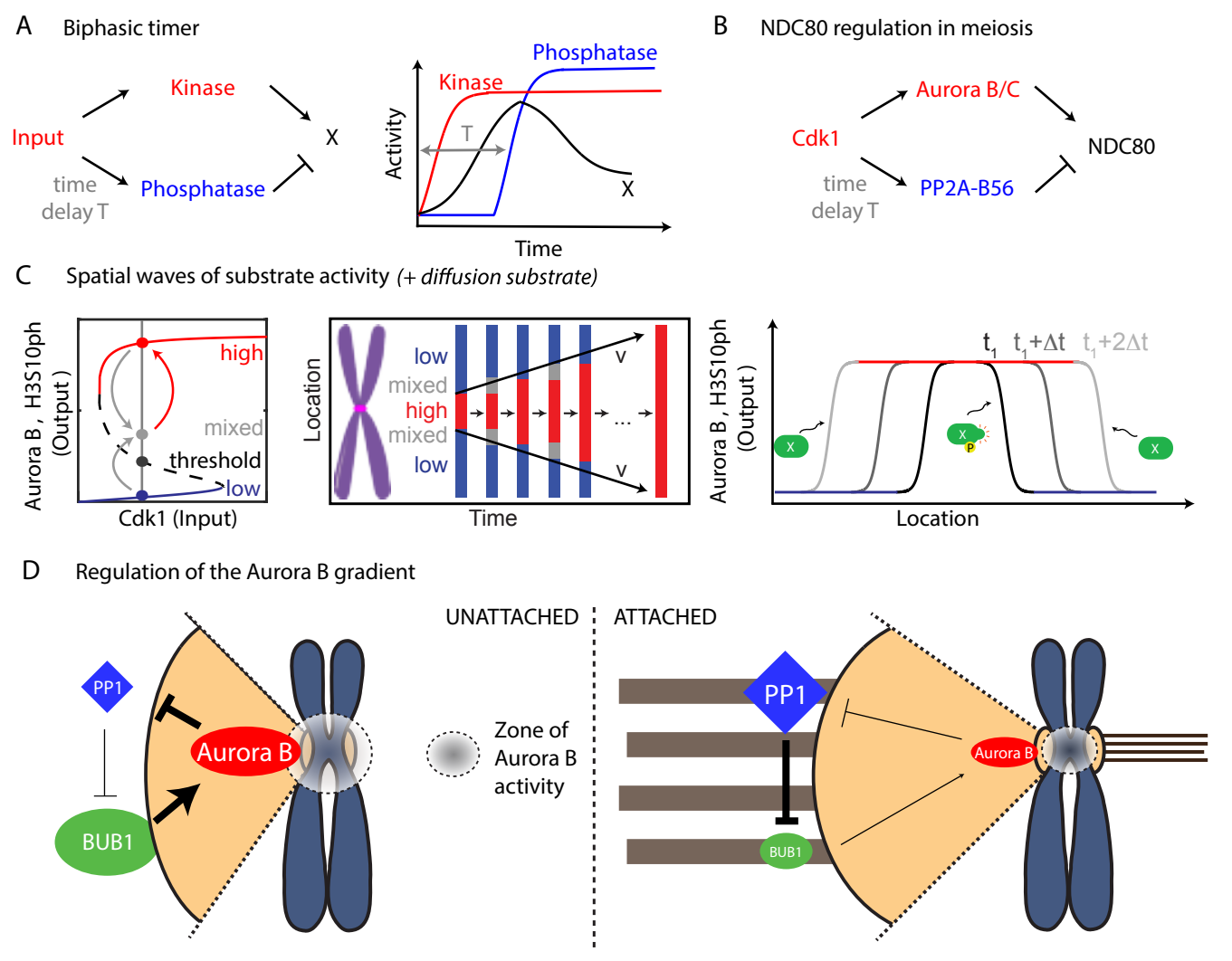
Other mitotic proteins

NDC80	Kinetochore protein that mediates load-bearing interactions with the spindle microtubules.
KIF4A	A kinesin-type motor protein that regulates the organization of the central spindle.
KNL1	A kinetochore-associated protein that functions as a scaffold for the assembly of the MCC complex and regulates microtubule-kinetochore interaction
Cohesin	Proteins that form a ring structure to keep the sister chromatids together.
Sororin	Protein that is required for the stable association of cohesin with sister chromatids.
Shugoshin (Sgo)	Centromeric protein that protects cohesion and promotes the recruitment of the CPC to the centromeres.
APC/C	Anaphase-promoting complex / Cyclosome is an E3 ubiquitin ligase that marks proteins for proteolytic degradation. It triggers the transition from metaphase to anaphase through the degradation of cyclin B and securin.
BUBR1	A pseudokinase that is part of the mitotic checkpoint complex (MCC); an inhibitor of the APC/C


A
Antagonistic regulation
Input1
Input2
X
Kinase
ATP
P
ADP
Phosphatase
X
P
Output
B
Time evolution: single vs. average response
X
P
X
Output
0
10
20
30
40
Time (a.u.)
C
Hyperbolic response
Output = Input / (K + Input)
100
50
0
Output (% phosph.)
0
1
2
3
4
Input
D
Ultrasensitivity
Output = Input^n / (K^n + Input^n)
n = 10
n = 5
n = 2
100
50
0
Output (% phosph.)
0
1
2
3
4
Input
E
Bistability
100
50
0
Output (% phosph.)
0
1
2
3
4
Input


A Spatial localisation of kinase and phosphatase
Phosphatase dominant
Kinase dominant
Phosphatase dominant
Kase / Ptase
Location
B Spatial localisation of substrate activity
(no diffusion substrate)
% phosph.
X
P
Location
E Spatial gradients of substrate activity
(+ diffusion substrate)
% phosph.
Location
C Centromeric cohesin
Aurora B
Cdk1
Plk1
Ssu72
Sgo1-PP2A
Ssu72
Kase dominant
Ptase dominant
Kase dominant
D Centromeric H3T3ph
Cdk1
Haspin
Aurora B
RepoMan/PP1
Ptase dominant
Kase dominant
Ptase dominant
F Spatial Pom1 gradient
Unphosph. Pom1 clusters at the cell tip
PP1
Tea
PP1
Tea
Microtubules
Cell center
PP1-Tea is transported to cell tip
Phosph. Pom1 diffuses away
Pom1 phosphorylation level
Low
High
Pom1 autophosphorylation
Pom1 transphosphorylation


A
Signal activation: 'off' to 'on'
Average phosph (%)
Time (a.u.)
no phosphatase
from time 0
from time t1
from time t2
t1
t2
B
Signal deactivation: 'on' to 'off'
Kinase high
Kinase low
Phosphatase low
Phosphatase high
C
AurB
MPS1
Prometaphase
(unattached kinetochore)
RVSF
MELT
KNL1
PP1
BUB3
BUB1
BUBR1
SAC
B56
PP2A
SAC signalling
(phosphatase primed to silence)
D
Prophase
(BUBR1 excluded from nucleus)
Nuclear envelope
Rapid signal start-up
(kinases unopposed)
Prometaphase
(attached kinetochore)
Rapid signal shut-down
(phosphatases silence quickly)
E
Thresholds
Output1
Output2
t1 ≠ t2
T1 = T2 = 0
K2
K1
Input
Time
F
Time delays
t1 = t2
T1 ≠ T2
T1
T2


A
Biphasic timer
Input
Kinase
time
delay T
Phosphatase
X
Activity
Kinase
Phosphatase
T
X
Time
B
NDC80 regulation in meiosis
Cdk1
Aurora B/C
time
delay T
PP2A-B56
NDC80
C
Spatial waves of substrate activity (+ diffusion substrate)
Aurora B , H3S10ph
(Output)
high
mixed
threshold
low
Cdk1 (Input)
Location
low
mixed
high
mixed
low
v
...
v
Time
Aurora B , H3S10ph
(Output)
t1 t1+Δt t1+2Δt
X
X
P
X
Location
D
Regulation of the Aurora B gradient
PP1
Aurora B
BUB1
UNATTACHED
Zone of
Aurora B
activity
ATTACHED
PP1
Aurora B
BUB1

Trends box

- Although individual phosphorylated molecules often behave like binary switches, multiple copies of each binary switch can be used to output complex biological responses.
- Kinase and phosphatases work together to shape how these multiple substrate copies behave in time and space.
- This 'cooperativity' is critical for determining three key properties of a signal response: amplitude, localisation and timing.
- These properties can be combined to generate complex outputs, such as spatial gradients (amplitude/localisation), biphasic responses (amplitude/time), dynamic signals (localisation/time), and spatial waves (amplitude/localisation/time).
- Understanding how kinase and phosphatase activities are integrated is therefore critical for defining how protein phosphorylation signals work.

Outstanding questions

- How do phosphatases achieve specificity for individual substrates, pathways and processes?
- Can we use this information to target phosphatases specifically?
- How do kinases and phosphatases cross-regulate each other?
- What are the scaffold proteins that mediate this cross-talk?
- How are kinase and phosphatase signals integrated to give the correct type of response?
- How do other 'antagonistic' enzyme pairs also cooperate to drive signalling processes?